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Stimulation of neutrophils by prenylcysteine analogs: Ca^{2+} release and influx

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Abstract

Farnesylthiosalicylic acid (FTS), a synthetic analog of the terminal prenylcysteine present in signaling proteins induces generation of superoxide ions, phospholipase C-driven hydrolysis of inositol lipids and calcium elevation in human neutrophils and DMSO-differentiated HL60 cells. These effects were ascribed to an interaction of the analog with elements responsible for recognition of specific prenylated proteins. The present study demonstrated that in addition to the release of intracellular calcium stores, FTS enhanced entry of Ca^{2+} and Mn^{2+} from the medium. The biphasic dependence of the influx on the concentration of FTS, as well as its insensitivity to inhibition by PMA and La^{3+} suggest that the influx pathway activated by FTS is distinct from the previously described store-operated calcium channels of neutrophils. Consistent with the participation of a cellular membrane component in the interaction, FTS enhanced ^{45}Ca uptake in neutrophils and neutrophil cell membranes, but not in multilamellar vesicles. To establish specificity of the farnesyl moiety of FTS (C_{15}), effects of three other analogs, geranylthiosalicylate, GTS (C_{10}), geranylgeranylthiosalicylate, GGTS (C_{20}), as well as the carboxymethyl ester FTS-Me on calcium homeostasis and superoxide production were investigated. GGTS dose-dependently elevated $[Ca^{2+}]_i$, induced quenching of the 360 nm Fura-2-calcium fluorescence by Mn^{2+} and stimulated superoxide release, while GTS and FTS-Me were inactive. These results defined specific structural requirements for the functional interaction of prenylcysteine analogs with myeloid cells.

Keywords: Neutrophil; HL-60 cell; Calcium homeostasis; Farnesylthiosalicylate; NADPH oxidase; Prenylcysteine analog

1. Introduction

Diverse signal transduction elements (small G-proteins, γ-subunits of heterotrimeric G-proteins, etc.) post-translationally undergo prenylation (farnesylation or geranylgeranylation) and methylation of their carboxyterminal cysteine residues. Prenylation (reviewed in [1,2]) enhances association of the modified

Abbreviations: PMN, polymorphonuclear leukocytes; FTS, farnesylthiosalicylate; GGTS, geranylgeranylthiosalicylate; GTS, geranylthiosalicylate; KRP, Krebs–Ringer phosphate solution; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid; fMLP, *N*-formyl-Met-Leu-Phe peptide; MLV, multilamellar vesicles; p.m., plasma membrane vesicles; PBS, phosphate-buffered saline; PC, phospatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-actetate; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; Tg, thapsigargin; AFC, *N*-acetyl-*S*-farnesylcysteine; PA, phosphatidic acid; DPPE, dipalmitoyl phospatidyl-ethanolamine

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proteins with membranes and facilitates protein–protein interactions [3].

Neutrophils constitute the first line of defense against invading microorganisms. They respond to exogenous signals by chemotaxis, activation of the respiratory burst NADPH oxidase, exocytosis of their cytoplasmic granules and phagocytosis of foreign particles [4,5]. Prenylated proteins implicated in stimulus–response coupling in activated neutrophils include geranylgeranylated Rac1/Rac2, Rho, Rap1A and γ-subunits of heterotrimeric G-proteins as well as farnesylated Ras.

Analogs of prenylcysteine evoke various responses in different cellular systems. We have recently reported [6] that, in neutrophils, farnesylthiosalicylic acid (FTS) induced NADPH oxidase-mediated formation of superoxide ions, phospholipase C-driven hydrolysis of inositol lipids and calcium elevation. In differentiated HL60 cells employed as a model of blood neutrophils, FTS enhanced activity of protein kinase C and of PMA-elicited NADPH oxidase. We suggested that activation by FTS may reflect interaction of the analog with prenyl recognition site/s on cellular components that participate in signal transduction.

In the present study, further characterization of the mechanism of calcium mobilization by FTS in myeloid cells was attempted with a special attention on the FTS-driven cation influx. Furthermore, specificity of the prenyl chain on superoxide production and calcium mobilization was studied by comparing effects of FTS (C_{15}) with effects of other prenylcysteine analogs, its carboxyl methyl ester, FTS-Me, geranylthiosalicylate, GTS (C_{10}) and geranylgeranylthiosalicylate, GGTS (C_{20}).

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma. FTS and the other prenylsalicylate analogs were synthesized as described previously [7,8] and solubilized in DMSO. Ionomycin was obtained from the Squibb Institute for Medical Research. ^{45}Ca was from New England Nuclear.

2.2. Cell culture

HL60 cells were grown in RPMI-1640 and induced to differentiation by DMSO as described [6].

2.3. Isolation and fractionation of neutrophils

Human neutrophils were isolated from fresh buffy coats by standard procedures of dextran sedimentation, gradient centrifugation on Lymphoprep (Nycomed, Norway) and hypotonic lysis of red blood cells. Cells (10^8/ml) were broken by sonication and fractionated by centrifugation as described [6]. Fractions were stored at −70°C.

2.4. Activation of the NADPH oxidase

Cells at 3×10^6 cells/ml were suspended in KRP (2 mM glucose, 131 mM NaCl, 5.2 mM KCl, 15.7 mM Na phosphate pH 7.4, 1.3 mM $MgSO_4$, 0.9 mM $CaCl_2$) containing 0.9 mg/ml of cytochrome *c* and stimulated. Superoxide generation was estimated as superoxide dismutase-inhibitable cytochrome *c* reduction at 550 nm in 96-well microplates (total volume of 0.1 ml) in the Thermomax (Molecular Devices) microplate reader.

2.5. Calcium measurements

Cells at 5×10^7 cell/ml in Ca^{2+}/Mg^{2+}-free KRP were loaded with 5 μM Fura-2AM at 37°C for 15 min, diluted with 4 vols. of Ca^{2+}/Mg^{2+}-free KRP and further incubated for 15 min. Cells were washed, resuspended in 0.8 ml KRP buffer at 2×10^6 cells/ml and the free calcium concentration was estimated in a Perkin–Elmer 44B spectrofluorometer by the method of Grynkiewicz et al. [9]. Excitation and emission wavelengths were 340 and 512 nm, respectively. Several experiments were carried out in the Perkin–Elmer LS50B spectrofluorometer in the 340/380 nm ratio mode [9]. Values of $[Ca^{2+}]_i$ reported in the text were taken from the maximum of each calcium transient, unless otherwise stated. Calcium-free medium consisted of Ca^{2+}/Mg^{2+}-free KRP supplemented with 1 mM EGTA.

2.6. Fura-2 fluorescence quenching by Mn^{2+}

Fura-2-loaded neutrophils in KRP were incubated with FTS for 1 min at 37°C. $MnCl_2$ (0.5 mM) was then added and rates of fluorescence quenching were monitored at 360 nm [10,11].

2.7. Preparation of multilamellar liposomes (MLV)

Phospatidylcholine (PC), azolectin or mixtures of PC, DPPE and cholesterol (4:4:2) or PC, PA and cholesterol (4:1:5) were dissolved in chloroform at 5 mg lipid/ml, evaporated and resuspended in phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl). Liposomes were pelleted before use by 5 min centrifugation at 11 000 × *g*.

2.8. ^{45}Ca uptake experiments

In a typical ^{45}Ca uptake experiment, the suspension of neutrophils (2×10^6/ml) or MLV (1 mM in lipid) in KRP (0.5 mM $CaCl_2$) was preincubated 5 min at 37°C in a total volume of 0.5 ml. FTS and ^{45}Ca (100 000 cpm) were then added and the incubation was continued for 5 min. The particles were then pelleted, washed (2×) by centrifugation and counted in a liquid scintillation counter. ^{45}Ca uptake experiments conducted with plasma membrane vesicles (80 μg protein/ml) in a 0.2 ml volume were terminated by centrifugation (100 000 × *g* 15 min) in the TLA 120.2 rotor of the L-100 Beckman ultracentrifuge at 4°C.

2.9. Statistical analysis

Experimental data are expressed as mean values ± S.E.M. unless otherwise stated. Representative experiments were repeated at least four times with similar results.

3. Results

3.1. Dose dependency of FTS-induced calcium elevation

In a previous communication we demonstrated FTS-induced production of IP_3, elevation of cyto-

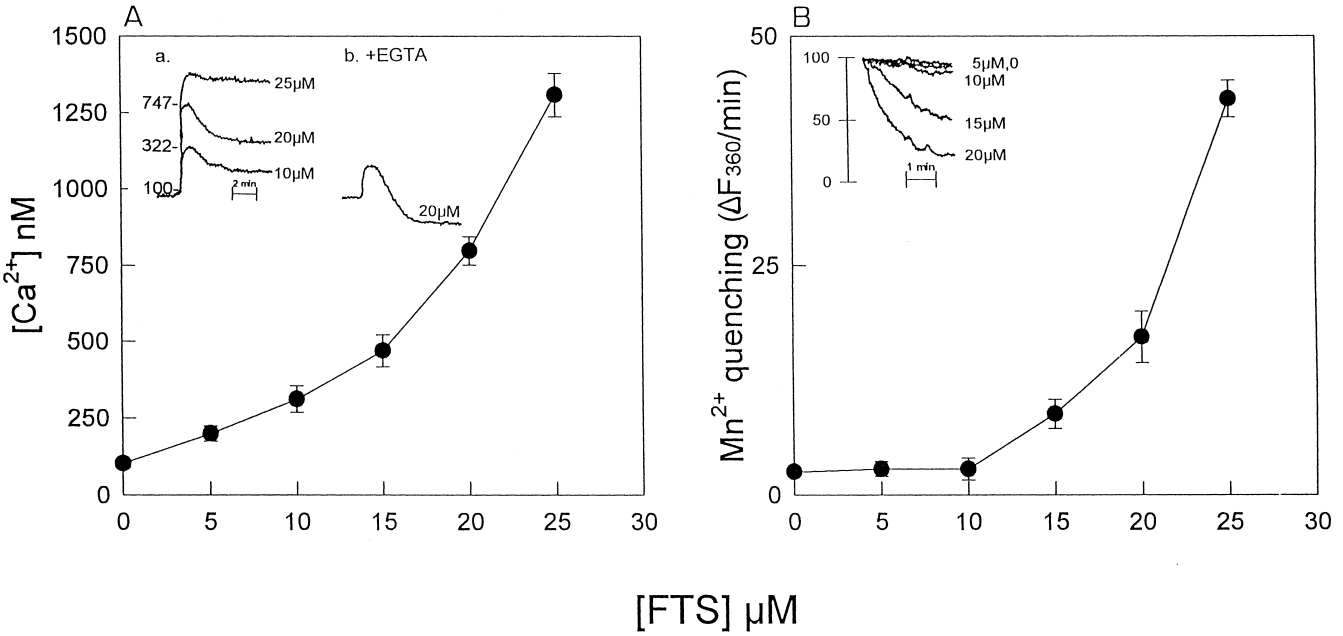

Fig. 1. The dependency of (A) $[Ca^{2+}]_i$ and (B) Mn^{+2}-elicited 360 nm fluorescence quenching rates on the concentration of FTS. (A) Insets: 340-nm fluorescence traces calcium transients in (a) Ca^{2+}-containing and (b) Ca^{2+}-free medium. (B) Quenching by Mn^{2+} was followed after the addition of 0.5 mM $MnCl_2$ (taken as 0 time) to FTS-prestimulated neutrophils. Inset: 360 nm fluorescence traces. Mean values ± S.E.M. for $n=5$ and $n=4$ experiments in A and B, respectively.

solic calcium and activation of the NADPH oxidase in neutrophils and HL60 cells. Elevation of $[Ca^{2+}]_i$ was ascribed to IP_3-mediated release of the ion from intracellular stores [6]. In the present study, the involvement of FTS in Ca^{2+} fluxes was further studied and a biphasic dose dependency of $[Ca^{2+}]_i$ on the concentration of FTS (5–25 μM) was observed. The increase in $[Ca^{2+}]_i$ was moderate below 15 μM and rose abruptly at 20–25 μM FTS (Fig. 1A). Trypan blue exclusion measurements indicated a considerable cytotoxicity above 30 μM FTS: mean values of $13.6 \pm 3.5\%$ and $34.5 \pm 5.2\%$ stained neutrophils were estimated at 30 and 40 μM FTS, respectively. In view of this, Ca^{2+} transients were determined only at the non-toxic doses of FTS.

Whereas addition of EGTA, to deplete the extracellular Ca^{2+}, reduced the FTS-elicited calcium signal (Fig. 1A, insets a and b), it did not affect the rate of superoxide release induced by FTS (6.3 ± 0.2 and 6.4 ± 0.25 nmol O_2^-/min in regular and in Ca^{2+}-free medium, respectively). These findings implied that activation of the NADPH oxidase by FTS was independent of the level of $[Ca^{2+}]_i$. In a calcium-free medium, the elevation of $[Ca^{2+}]_i$ by 20 μM FTS was transient and the signal declined thereafter below its levels in resting neutrophils and HL60 cells (Fig. 1A, inset b). This reduction in $[Ca^{2+}]_i$ was compatible with either a reuptake of the ion into calcium-depleted stores or with its accelerated efflux into the medium. Ionomycin which permits evaluation of the contents of the residual calcium pool [11] was used to distinguish between these possibilities. Since the addition of ionomycin to cells treated with FTS in a Ca^{2+}-free medium did not elicit elevation of $[Ca^{2+}]_i$ (data not shown), refilling of stores was ruled out suggesting efflux as the more likely explanation.

3.2. The effect of FTS on influx of cations from the medium

Emptying of cellular stores is thought to induce entry of Ca^{2+} from the medium through non-specific, voltage-independent cation channels (store-operated calcium channels) [11–13]. The sustained increase in $[Ca^{2+}]_i$ observed at 20–25 μM FTS (Fig. 1A, inset a) was compatible with participation of an influx component in calcium homeostasis. In many types of cells, including neutrophils, Mn^{2+} ions act as a surrogate of Ca^{2+} entering the cell through identical channels [10,11,14]. Quenching of the 360 nm fluorescence of calcium-bound Fura-2 by Mn^{2+} permits fluorometric monitoring of Mn^{2+} uptake by the dye-loaded cells. As shown in Fig. 1B unstimulated neutrophils (or HL-60 cells, not shown) exhibited a low

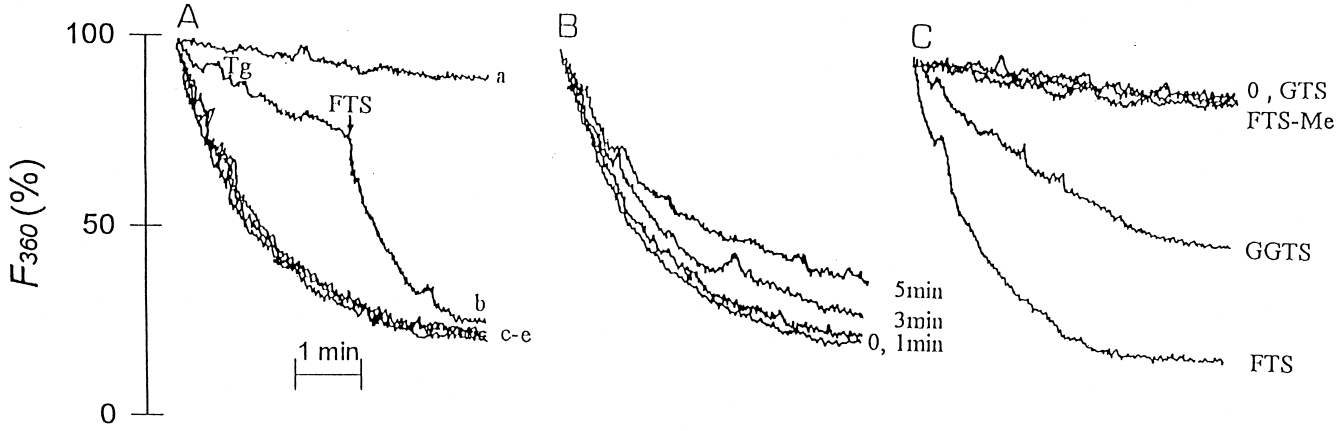

Fig. 2. Mn^{2+} quenching traces obtained upon the addition at 0 time of 0.5 mM $MnCl_2$ to Fura-2-loaded neutrophils. Fluorescence quenching was monitored at 360 nm. (A) Trace a, untreated control; trace b, cells preincubated with Tg (50 nM, 5 min) before the addition of $MnCl_2$ and stimulated as indicated with FTS; trace c, cells pretreated (1 min) with FTS; traces d and e, cells preincubated with La^{3+} (0.2 mM) or PMA (100 ng/ml) for 1 min before stimulation with FTS. (B) Fluorescence traces of neutrophils incubated with FTS for 0, 1, 3 or 5 min prior to the addition of $MnCl_2$. (C) Fluorescence traces in neutrophils preincubated (1 min) prior to the addition of Mn^{2+} with FTS, FTS-Me, GGTS or GTS (20 μM); 0, unstimulated cells. Representative experiments repeated four times with similar results.

rate of 360 nm fluorescence quenching by $MnCl_2$; this low rate was retained at low concentrations of FTS and increased markedly at higher doses, consistent with the biphasic response of $[Ca^{2+}]_i$ (Fig. 1A). It is of note that the lower doses of prenylcysteine analogs, ineffective in Mn^{2+} quenching, elicited activation of neutrophil NADPH oxidase [6] as well as store-mediated transient elevation of intracellular calcium concentration (Fig. 1A). La^{3+} ions, shown elsewhere to block the entry of Mn^{2+} and Ca^{2+} into neutrophils [14], did not affect FTS-induced Mn^{2+} entry (Fig. 2A, trace d) implying that FTS might have activated a distinct Ca^{2+} inflow pathway.

In neutrophils, rates of fluorescence quenching decreased slightly when 5 min elapsed between stimulation with FTS and the addition of Mn^{2+} (Fig. 2B). In HL-60 cells, the duration of incubation with FTS (0–5 min) prior to the addition of $MnCl_2$ had no effect on rates of Mn^{2+} uptake (data not shown) implying that influx of Mn^{2+} was independent of $[Ca^{2+}]_i$.

Phorbol esters were shown to block store-operated cation channels of myeloid cells [15]. In our case, however, the level of FTS-elicited $[Ca^{2+}]_i$ as well as the rate of Mn^{2+} inflow were not altered by 1 min preincubation with PMA (Fig. 2A, trace e).

3.3. *Thapsigargin (Tg)- and FTS-releasable intracellular calcium stores*

Tg, an inhibitor of intracellular Ca-ATPase, induces a sustained elevation of cytosolic calcium concentration by an indirect and irreversible depletion of cellular stores [16]. In several types of cells, including neutrophils and HL60 cells [12–15], Tg opens store-operated channels in the cell membrane for influx of Ca^{2+} and Mn^{2+} from the medium. Neutrophils pretreated with a saturating dose of Tg (50 nM, 5 min) in a Ca^{2+}-containing medium responded to 20 μM FTS by a secondary raise in $[Ca^{2+}]_i$ (Fig. 3, upper trace). A small and transient calcium elevation was also induced by FTS in Tg-treated cells suspended in a calcium-free medium (Fig. 3, lower trace), implicating a residual Tg-insensitive intracellular calcium pool. The reciprocal treatment, namely addition of Tg to cells preexposed to FTS did not elicit any response, implying that whereas FTS emptied Tg-sensitive stores, only a fraction of FTS-susceptible stores was released by Tg (not shown). Compatible with this finding, in cells pre-exposed to Tg, FTS markedly accelerated Mn^{2+}-elicited fluorescence quenching (Fig. 2A, trace b).

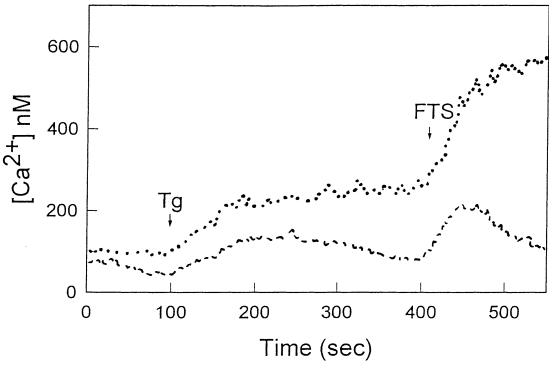

Fig. 3. FTS-induced calcium elevation in Tg-pretreated (50 nM, 5 min), Fura-2-loaded neutrophils; upper trace, calcium containing; lower trace, calcium-free medium. A representative experiment repeated six times with similar results.

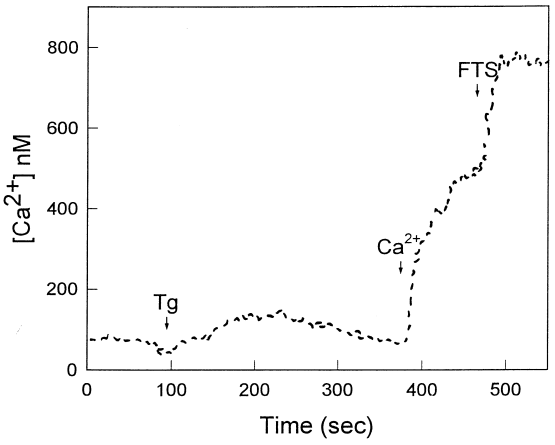

Fig. 4. The effect of exogenous Ca^{2+} (1.2 mM) followed by FTS on $[Ca^{2+}]_i$ in Tg-pretreated (50 nM, 5 min) neutrophils in a calcium-free medium. A representative experiment repeated four times with similar results.

As previously shown [17], the addition of 1.2 mM $CaCl_2$ to neutrophils pretreated with Tg in a calcium-free medium evoked a rapid raise in $[Ca^{2+}]_i$ due to inflow of the ion through the open store-operated channels in the plasma membrane (Fig. 4). Subsequent application of FTS resulted in an additional and substantial increase in $[Ca^{2+}]_i$. These observations corroborate our conclusion that in addition to the release of stored calcium FTS opens or modulates activity of a calcium influx pathway.

3.4. Cells pretreated with FTS respond to a secondary stimulus

Although Trypan blue exclusion and retention of cytosolic lactate dehydrogenase (data not shown) indicated that pretreatment of cells with 20 μM FTS (5 min, 37°C) did not affect their viability, the pronounced enhancement in Ca^{2+} entry elicited by FTS (Fig. 1A) prompted us to test the effect of FTS on the leakage of Fura-2 from dye-loaded neutrophils. For this purpose FTS-treated and control cells were pelleted and the dye released into the supernatant was fluorometrically estimated. Untreated and FTS-treated neutrophils released 17 ± 3.2% and 18 ± 4.4% of their total Fura-2, detected in Triton X-100 lysate of the loaded cells, respectively. Similar values were obtained in HL-60 cells (21 ± 4.5% and 21.3 ± 4.2% respectively). These results imply in spite of the changes in Ca^{2+} and Mn^{2+} permeability, the FTS-stimulated cells were not leaky.

It was of interest whether removal of exogenous FTS by centrifugation restores responsiveness of the cells with respect to their $[Ca^{2+}]_i$ and superoxide production. For determination of Ca^{2+}, Fura-2 loaded neutrophils were pretreated with 20 or 25 μM FTS (5 min, 37°C), pelleted and resuspended at 37°C in the absence of FTS. Levels of $[Ca^{2+}]_i$ determined in the prestimulated and resuspended HL60 cells and in unstimulated controls were 160 ± 45 and 89.5 ± 25 nM, respectively. In neutrophils somewhat higher levels of Ca^{2+} were observed (340 ± 58 nM compared to 117.7 ± 16 nM of untreated cells). When FTS present during the pretreatment was replaced by 100 nM ionomycin, the elevated levels of $[Ca^{2+}]_i$ were retained (above 700 ± 45 nM).

Restimulation of FTS-pretreated cells with FTS initiated a very prominent and sustained secondary elevation of $[Ca^{2+}]_i$. To determine activity of the NADPH oxidase in response to restimulation with FTS, the FTS-pretreated (20 μM, 5 min) and pelleted neutrophils were resuspended and divided into two aliquots: in the first (control) aliquot, the residual superoxide release rate was estimated (11.73 ± 1.8 nmol/5 min). Restimulation of the second aliquot with FTS resulted in a significantly higher secondary superoxide response (24.1 ± 2.03 nmol/5 min) indicating that elements participating in activation of the NADPH oxidase increased their responsiveness to FTS.

3.5. ^{45}Ca uptake by FTS-stimulated cells and liposomes

To establish whether FTS affects Ca^{2+} and Mn^{2+} influx by interaction with a specific membrane component ^{45}Ca uptake by three different types of membranes was compared. Neutrophils, neutrophil plasma membrane vesicles and multilamellar liposomes were pretreated with increasing concentrations of FTS in the presence of ^{45}Ca. Particle-associated ^{45}Ca was then determined and compared with corresponding values obtained by permeabilization of each type of membrane by ionomycin (1 μM) (Fig. 5). As shown in Fig. 5, FTS enhanced association of ^{45}Ca with the cells at 20 μM, but not below this concentration. A similar effect of FTS on ^{45}Ca inflow was observed employing neutrophil plasma membrane vesicles (Fig. 5). Contrary to these results FTS (up to 25 μM) did not enhance ^{45}Ca uptake by phosphatidylcholine multilamellar liposomes. Likewise, 20 μM FTS did not induce ^{45}Ca uptake into three types of multilamellar vesicles of different

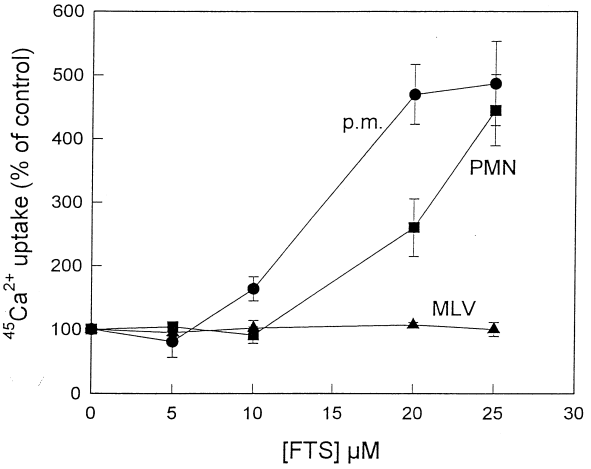

Fig. 5. The dependency of ^{45}Ca uptake by neutrophils (PMN) (2×10^6/ml), PC multilamellar vesicles (MLV) (1 mM lipid) or plasma membrane vesicles (p.m.) (80 μg protein/ml) on the concentration of FTS (5 min, 37°C). Control, unstimulated particles. Ionomycin-induced ^{45}Ca uptake (in percent of control): PMN, 575 ± 110; p.m., 702 ± 112; MLV, 324.6 ± 89.3%). Detailed procedures are described in Section 2. Mean values ± S.D. for $n = 4$.

lipid composition, azolectin, PC:DPPE:cholesterol (4:4:2) or PC:PA:cholesterol (4:1:5) (the uptake did not exceed 115% of the untreated controls).

3.6. Elevation of intracellular calcium concentration by FTS and GGTS

To define specificity of the isoprenoid moiety, effects of several analogs of FTS on calcium homeostasis were compared. Geranylgeranylthiosalicylate (GGTS), dose-dependently elevated $[Ca^{2+}]_i$, exerting somewhat lower potency than FTS. Geranylthiosalicylate (GTS), the carboxymethyl ester of FTS (FTS-Me) [8] as well as *N*-acetyl-*S*-farnesylcysteine (AFC) had no effect on calcium concentration (Table 1). GGTS resembled FTS also in quenching of the 360 nm Fura-2-calcium fluorescence by Mn^{2+} (Fig. 2C), whereas GTS and Me-FTS did not alter the basal uptake of Mn^{2+} in neutrophils and HL-60 cells.

3.7. FTS and GGTS, but not FTS-Me or GTS, activate neutrophil NADPH oxidase

Effects of the prenylsalicylates on activation of neutrophil NADPH oxidase are summarized in Table 1. Their relative activities on the superoxide-generating enzyme resembled their effects on Ca^{2+} signals: FTS and GGTS were potent inducers of the NADPH oxidase whereas GTS, FTS-Me and AFC were ineffective. A similar order of potencies was observed in case of PMA-induced activation of the oxidase in HL60 cells [6] (data not shown).

Table 1
Effects of FTS analogs on superoxide release and on cytosolic $[Ca^{2+}]$

	Superoxide release (nmol O_2^-/min)	$[Ca^{2+}]_i$ (nM)
Basal	0.1 ± 0.0	104 ± 11.6
GTS	0.3 ± 0.2	100 ± 12.0
FTS	6.3 ± 0.2	765 ± 35.4
GGTS	2.6 ± 0.1	427 ± 11.6
FTS-Me	0.2 ± 0.1	99 ± 8.7
AFC	0.1 ± 0.1	104 ± 24.9

The experimental details are described in Section 2. Mean values ± S.E.M. ($n = 5$).

4. Discussion

We have recently reported that the prenylcysteine analog FTS elicited activation of the neutrophil NADPH oxidase and released calcium from intracellular stores of myeloid cells [6]. Consistent with a partial dependence on G_i proteins, both effects were in part pertussis toxin sensitive. In the present report, we further characterized the FTS-sensitive calcium stores and studied prenylcysteine analog-mediated influx of calcium and NADPH oxidase activation. We showed that neutrophils pretreated with Tg responded to FTS by an additional elevation of their $[Ca^{2+}]_i$ in a calcium-containing as well as in a calcium-free medium (Fig. 3). Since Tg defined as an inhibitor of the intracellular Ca-ATPase irreversibly empties calcium stores [16], this finding provides evidence for the existence in myeloid cells of Tg-insensitive stores, replenished by a Ca-ATPase distinct from the well-characterized SERCA pump of endoplasmic reticulum [16,18]. Although Tg-insensitive pools have been documented in several types of cells [19,20], their presence in myeloid cells was reported only in the case of stimulation by very-long-chain fatty acids [21]. It remains to be seen whether the FTS-releasable Tg-insensitive residual stores bear any relationship to the peripheral integrin-linked calcium pools described recently by Pettit and Hallett [22].

Another feature characterizing the prenylthiosalicylates-mediated calcium signaling described in the current communication involves the enhanced influx of Ca^{2+} from the medium. This influx was responsible for the sustained and pronounced $[Ca^{2+}]_i$ elevation elicited by higher concentrations of FTS (20–25 μM) in a calcium-containing medium. In the absence of extracellular calcium, $[Ca^{2+}]_i$ elevation was transient, falling progressively below its level in the resting cell. Since at these conditions application of ionomycin, expected to release the full content of calcium stores [11] did not elicit any additional Ca^{2+} signal, it may be suggested that the fall in $[Ca^{2+}]_i$ subsequent to the addition of FTS reflected an enhanced efflux of the ion into the medium.

Inflow of Ca^{2+} from the medium through store-operated channels in the plasma membrane of myeloid cells has been extensively studied [10–12,14,15,17]. The theory of capacitative calcium en-

try correlates opening of non-specific cation channels in the cell membrane with a previous depletion of intracellular calcium stores [13,23]. Since Mn^{2+}, acts as Ca^{2+} surrogate flowing into the cell through these channels, the Mn^{2+} quench method was used for evaluation of the contribution of the influx component to changes in $[Ca^{2+}]_i$. At low FTS concentrations the elevation of $[Ca^{2+}]_i$ was gradual and transient and the rate of Mn^{2+} entry remained low (Fig. 1), consistent with a release of Ca^{2+} from intracellular stores. Both Ca^{2+} and Mn^{2+} inflow rates increased markedly at higher FTS (>15 μM) exhibiting a positive cooperativity. This result is compatible with an influx pathway interacting at several sites with FTS or with self-association of FTS molecules.

Several features of Ca^{2+} homeostasis established in the Mn^{2+} quench experiments appear incompatible with the capacitative theory. Firstly, in contrast to store-operated calcium channels of myeloid cells, La^{3+} described as an efficient calcium channel blocker [14] had no effect on Mn^{2+} entry (Fig. 2A, trace d). Secondly, the rate of FTS-stimulated Mn^{2+} uptake through these channels, reportedly downregulated by protein kinase C [15,17], was not altered by preincubation with PMA (Fig. 2A, trace e). Thirdly, according to the capacitative theory, store emptying precedes influx from the medium while in the case of FTS, the high rate of Mn^{2+} uptake was apparent immediately on the addition of $MnCl_2$ (Fig. 1B) without a measurable delay required for emptying the intracellular pool. It should be kept in mind, however, that very short latencies preceding influx have also been described [11–13,23]. Notwithstanding the listed above inconsistencies with capacitative calcium entry the FTS-treated cells were not leaky since they excluded Trypan blue, retained Fura-2 and were capable of a secondary stimulation of the NADPH oxidase which depends on the retention of cytosolic NADPH and GTP.

The observation that methylation of the free carboxyl group of FTS in FTS-Me abrogates activity of the compound in NADPH oxidase activation and in calcium signaling (Table 1, Fig. 2C) raised the possibility that the carboxyl group of FTS participated in calcium ion transport by acting as an ionophore. Several lines of evidence argue against this hypothesis: (1) in contrast to cells and membrane vesicles, FTS-treated MLV of varying lipid composition did not exhibit enhanced association with ^{45}Ca (Fig. 5 and the text); (2) unlike ionomycin, FTS was easily removed from the cells by centrifugation; (3) DMSO-induced HL-60 cells grown for 5 days in the presence of 20 μM FTS (6) exhibited a normal course of differentiation (unpublished results). Moreover, the functional specificity of the isoprenoid moiety in FTS and GGTS compared to GTS (see below) and the lack of calcium-elevating activity of the farnesyl analog AFC which contains a free carboxyl group (Table 1, Fig. 2C) support a specific interaction of the prenyl moiety with a membrane component that modulates Ca^{2+} transport. A complex of this kind might participate in FTS-stimulated cation entry as a Ca^{2+} influx pathway distinct from the store-operated calcium channel.

Participation of trimeric G-proteins in store-dependent Ca^{2+} influx into myeloid cells has been suggested by Jaconi et al. [24]. Evidence in favor of a small G-protein has been presented in rat basophilic leukemia cells [25] and Rac proteins have recently been implicated in growth factor-dependent transport of Ca^{+2} into cells [26]. Xu et al. reported blockade of the capacitative Ca^{2+} entry into embryonic kidney 293 cells by the farnesylcysteine analogs *S*-farnesylthioacetic acid (FTA), *N*-acetyl-*S*-farnesylcysteine (AFC) and *N*-acetyl-*S*-geranylgeranylcysteine (AGGC) [27]. Their photoaffinity labeling experiments utilizing an analog of farnesylcysteine pointed to the participation of a small prenylated G-protein in the capacitative Ca^{2+} inflow. It is noteworthy that in contrast to the NADPH oxidase-stimulating and calcium-elevating effects of FTS and GGTS described in the current study, the capacitative Ca^{2+} entry-blocking analogs employed by Xu et al. interfered with fMLP-elicited superoxide release in neutrophils [28]. In our hands, activation of the neutrophil NADPH oxidase by FTS took place, however, also in the absence of extracellular calcium, implying dissociation of calcium inflow from the superoxide response. It follows that the basis of the correlation between effects of prenylcysteine analogs on calcium entry and on oxidase activation ([26,27] and the present study) remains to be elucidated.

Although the pathway involved in prenylthiosalicylate-induced inflow of Ca^{2+} and Mn^{+2} differs in its properties from regular store-operated cation channels, it may be regulated by a small prenylated G-

protein. Most likely candidates for such a function are Rac and Rap GTP-binding proteins. Rac protein constitutes an essential component of the neutrophil NADPH oxidase [4] and has previously been implicated in Ca^{2+} transport in cells [26]. Rap1A is expressed in neutrophils and has been implicated in the regulation of the NADPH oxidase [29]. Activation of neutrophil Rap1A by an increased $[Ca^{2+}]_i$ has recently been demonstrated [30]. It is conceivable that Ca^{2+} released from intracellular pools may activate Rap1 and that the activated Rap1 may support further inflow of Ca^{2+} from the medium by a calcium-induced calcium entry mechanism. Further studies will be required to substantiate these possibilities.

Since prenylated proteins may be either farnesylated or geranylgeranylated, the effectiveness of GGTS, FTS and GTS to mobilize calcium and activate respiratory burst oxidase was compared (Table 1). In both functional tests, the farnesyl group followed by the geranylgeranyl group exhibited optimal structures for activity whereas the 10-carbon isoprenoid moiety of geranylthiosalicylate was inactive. Interestingly, similar relative potencies have recently been reported by Aharonson et al. in the case of prenylthiosalicylate-mediated inhibition of Ras-dependent growth of fibroblasts [31]. In their systems, the active analogs affected membrane anchorage of activated H-Ras and blocked Ras-mediated signaling [7,32]. It is remarkable that structural elements that promote interference with growth-linked functions of activated Ras and induce dislodgement of Ras in fibroblasts, mediate ion fluxes and activation of the NADPH oxidase in myeloid cells.

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